Streptococcus oralis maintains homeostasis in oral biofilms by antagonizing the cariogenic pathogen Streptococcus mutans

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Abstract: Bacteria residing in oral biofilms live in a state of dynamic equilibrium with one another. The intricate synergistic or antagonistic interactions between them are crucial for determining this balance. Using the six-species Zürich "supragingival" biofilm model, this study aimed to investigate interactions regarding growth and localization of the constituent species. As control, an inoculum containing all six strains was used, whereas in each of the further five inocula one of the bacterial species was alternately absent, and in the last, both streptococci were absent. Biofilms were grown anaerobically on hydroxyapatite disks, and after 64 h they were harvested and quantified by culture analyses. For visualization, fluorescence in situ hybridization and confocal laser scanning microscopy were used. Compared with the control, no statistically significant difference of total colony-forming units was observed in the absence of any of the biofilm species, except for Fusobacterium nucleatum, whose absence caused a significant decrease in total bacterial numbers. Absence of Streptococcus oralis resulted in a significant decrease in Actinomyces oris, and increase in Streptococcus mutans ($P < .001$). Absence of A. oris, Veillonella dispar or S. mutans did not cause any changes. The structure of the biofilm with regards to the localization of the species did not result in observable changes. In summary, the most striking observation of the present study was that absence of S. oralis resulted in limited growth of commensal A. oris and overgrowth of S. mutans. These data establish highlight S. oralis as commensal keeper of homeostasis in the biofilm by antagonizing S. mutans, so preventing a caries-favoring dysbiotic state.

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Streptococcus oralis maintains homeostasis in supragingival biofilms by antagonizing cariogenic pathogen *Streptococcus mutans*

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Summary

Bacteria residing in oral biofilms live in a state of dynamic equilibrium with one another. The intricate synergistic or antagonistic interactions between them are crucial for determining this balance. Using the 6-species Zürich "supragingival" biofilm model, this study aimed to investigate interactions regarding growth and localization of the constituent species. As control, an inoculum containing all six strains was used, whereas in each of the further five inocula one of the bacterial species was absent, and in the last both streptococci were absent. Biofilms were grown anaerobically on hydroxyapatite discs, and after 64 h they were harvested and quantified by culture analyses. For visualization, fluorescence *in situ* hybridization and confocal laser scanning microscopy was used. Compared to the control, no statistically significant difference of total CFU was observed in the absence of any of the biofilm species, except for *F. nucleatum* whose absence caused a significant decrease in total bacterial numbers. Absence of *S. oralis* resulted in a significant decrease in *A. oris*, and increase in *S. mutans* ($p<0.001$). Absence of *A. oris*, *V. dispar* or S. *mutans* did not cause any changes. The structure of the biofilm with regards to the localization of the species did not result in observable changes. In summary, the most striking observation was that absence of *S. oralis* resulted in limited growth of commensal *A. oris* and overgrowth of *S. mutans*. This data establishes *S. oralis* as commensal keeper of homeostasis in the biofilm by antagonizing *S. mutans*, thus preventing a caries-favoring dysbiotic state.

Introduction

Despite their microbial diversity, dental biofilms display a high homeostatic capacity that allows them to metabolically adapt to changing environmental conditions and exposures to stresses, such as the host defenses, diet, invasion of exogenous species, antimicrobial agents, and changes in salivary flow or hormone levels. Metabolic exchange occurs bi-directionally between species. For instance, lactic acid produced by streptococci, such as *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus gordonii* promotes the use as a source of energy for growth by *Veillonella* sp [1] or *Aggregatibacter actinomycetemcomitans* [2]. The interactions between species in a biofilm enable them to grow, survive and find their own metabolic role(s) within their polymicrobial community.

An efficient way to study inter-species interactions is with the help of *in vitro* grown biofilms consisting of multiple oral species. Such is the Zürich biofilm model in its supragingival [3, 4], subgingival [5], or transitional form [6]. The microorganisms comprising this *in vitro* model have been selected as typical of supragingival dental plaque, grown in a batch culture approach on salivary pellicle-coated hydroxyapatite discs [7]. The reproducibility, composition, 3D-structure and functional properties, such as mass transport of macromolecules and demineralization properties have been thoroughly characterized in earlier studies [7–12]. This standardized and highly reproducible model is useful in testing the antimicrobial capacity of mouthrinses [7, 13], interactions with host tissues/cells [14], and interactions between species within species. For instance, in recent studies we demonstrated the effects of adding exogenous species in the supragingival biofilm, showing that *Enterococcus faecalis* and *Staphylococcus aureus* can survive and grow like endogenous species, whereas *Eschericia coli* can predominate in the biofilm under the experimental conditions [15].

We have also studied the microbial dynamics that take place during the conversion from supragingival to subgingival conditions [6]. Accordingly, in the subgingival model we have shown that omission of the "early colonizing" species (e.g. streptococci and actinomyces) did not hinder biofilm formation, yet results in considerable changes in the bioflm's microstructure [16]. Moreover, depletion of species-specific virulence factors can also result in structural changes in the biofilm [17, 18].

There is a need to study the individual effects of each one species in the biofilm community, in order to drive more solid conclusions on their relative roles. The present study was designed to address this need, using the 6-species Zürich "supragingival" biofilm model, aiming to compare biofilms lacking each one of species to the standard 6-species biofilm control. The studied outputs were bacterial numbers (measured as colony forming units; CFUs) and visual localization of selected species in the biofilm (evaluated by confocal laser scanning microscopy; CLSM) in this supragingival biofilm model.

Materials and methods

Formation of *in vitro* supragingival biofilm

The standard six-species supragingival biofilm employed contained *Actinomyces oris* OMZ 745, *Candida albicans* OMZ 110, *Fusobacterium nucleatum* OMZ 598, *Streptococcus mutans* OMZ 918, *Streptococcus oralis* OMZ 607, and *Veillonella dispar* OMZ 493. Seven different inocula were used: As a control, an inoculum containing all six strains was used, whereas in each of the further five inocula one of the bacterial species was absent, and in the last inoculum both streptococci were absent. The influence of the absence of *C. albicans* was not tested. The procedure to

produce biofilms has been described previously [7, 9, 19]. In brief, Biofilms were grown anaerobically in 24-well culture dishes on hydroxyapatite discs that had been preconditioned for pellicle formation in whole un-stimulated pooled saliva (in the following termed saliva) for 4 h. To initiate a biofilm experiment disks were covered for the first 16 h with 1.6 ml of growth medium containing 70% saliva, 30% modified fluid universal medium (mFUM) [3] supplemented with Sørensen's buffer (final pH 7.2) and 200 µl of a cell suspension prepared from equal volumes and densities of each strain. The medium was changed after 16 h and 40 h. For the first 16 h, the medium contained 0.3% glucose. After 16 h the medium was replenished with one containing 0.15% glucose and 0.15% sucrose, instead of 0.3% glucose. In order to remove non-adherent micro-organisms, biofilms were dipped 3x in saline after 16, 20 and 24 h as well as after 40, 44 and 48 h. After 64 h incubation, the biofilms were dip-washed again before harvesting.

Quantitative determination of biofilm species

After 64 h of biofilm growth, the hydroxyapatite discs were vortexed vigorously for 1 minute in 1 ml of 0.9% NaCl to harvest the adherent biofilms. After vortexing the harvested biofilms were sonicated, to ensure that the bacteria were dispersed. The resulting bacterial suspensions were serially diluted in 0.9% NaCl. Of each serial dilution, 50 µl aliquots were plated on Columbia blood agar base (Oxoid Ltd., Basingstoke, UK) supplemented with 5% whole human blood (CBA) to estimate total colony-forming units (CFUs). To determine the species-specific bacterial numbers, selective agars were used to determine the CFUs for the species of the biofilms as described earlier [3, 20]. In brief, CBA plates were used to obtain total bacterial counts and to enumerate *A. oris* and *V dispar*; differential counting of *S. mutans* and *S. oralis*

was accomplished with the use of Mitis Salivarius Agar (Difco Laboratories, Inc., Detroit, MI, USA) supplemented with 0.001% (w/v) Na tellurite, whereas selective growth of *F. nucleatum* was achieved with Fastidious Anaerobe Agar (Chemie Brunschwig, Basel, Switzerland) and BIGGY Agar (BBL, BD Diagnostic Systems) was used to enumerate *C. albicans*. Agar plates were incubated at 37°C for 72 h. Species identification was achieved by observation of colony morphology.

Staining of biofilms by fluorescence in situ hybridization (FISH)

The biofilms were stained by FISH following earlier described protocols [10, 21]. In brief, pre-hybridization (15 min, 46 °C) was performed in 500 µl hybridization buffer in the absence of any oligonucleotide probes. Thereafter, 500 µl of hybridization buffer was used for each biofilm, supplemented with genus or species specific probes at a concentration of 20 ng/µl. The incubation time for the hybridization was at least 3 h at 46 °C in the dark. After the incubation, biofilms were transferred into washing buffer pre-heated to 48 °C and incubated for 20 min at this temperature. For counterstaining, biofilms were stained using a mixture of 3 µM YoPro 1 iodide (Invitrogen) and 15 µM Sytox green (Invitrogen) (20 min, room temperature, in the dark), following the FISH procedure. After staining, the samples were embedded upside-down on chamber slides in 100 µl of Mowiol [4].

Visualization of the biofilms by confocal laser scanning microscopy (CLSM)

Stained biofilms were examined by CLSM using a Leica TCS SP5 microscope (Leica Microsystems) with a x100/1.4 NA oil immersion objective lens, in conjunction with an Argon laser at 488 nm excitation, a DPSS diode laser at 561 nm, and a Helium-Neon laser at 633 nm excitation. Filters were set to 500–540 nm for YoPro/Sytox, to

570–600 nm for Cy3, and to 660–710 nm for Cy5, respectively. Biofilms were scanned in sequential mode and z-series were generated by vertical optical sectioning using a step size of 1 μm. Image acquisition was done in x8 line average mode and scans were recombined and processed using IMARIS 7.6.5 software (Bitplane, Zurich, Switzerland), without any qualitative changes to the raw images.

Statistical analysis

Three individual experiments were performed and each group represented in triplicate biofilm cultures per experiment. A two-way analysis of variance (ANOVA) in conjunction with Tukey's multiple comparison text was used to evaluate the differences between the control and each experimental group. The significance level was set to $P < 0.05$. Values below the assay's detection limit where ascribed the lowest detection limit value, to allow for logarithmic transformation. The Prism v.6 statistical analysis software (GraphPad, La Jolla, CA) was used to analyze the data.

Results

At first, the total CFUs and the CFUs of each individual species were considered (Figure 1). Compared to the standard control biofilm, only the absence of *F. nucleatum*, a "bridging" biofilm species, caused a significant ($p<0.05$) reduction in total species CFU numbers. Nevertheless, the absence of *S. oralis* resulted in a significant decrease in *A. oris*, along with a significant increase in *S. mutans* ($p<0.001$), the latter increasing from from $1.2x10^7$ to $2.0x10^8$ CFUs. On the other hand, the absence of *A. oris*, *V. dispar* or S. *mutans* did not affect the numbers of any of the individual species. Interestingly, absence of both *S. oralis* and *S. mutans* from the biofilm results in potentiation further the growth of *A. oris*, collectively indicating

that *S. mutans* exerted an inhibitory effect. Although *C. albicans* was not omitted from the biofilms, it was shown that it was able to overgrow when the two streptococci were absent, indicating a competitive relationship with regards to growth and/or space.

The localization of selected species whose numbers were affected was also studied. In the standard 6 species biofilm, *A. oris* was distributed in small sparse clusters, whereas *F. nucleatum* more evenly intertwined throughout the biofilm (Figure 2A). In the absence of the both *S. mutans* and *S. oralis*, the allocation of *F. nucleatum* remained unchanged, whereas the small clusters of *A. oris* became multiple and denser (Figure 2B), corroborating the numeric increase of *A. oris* (Figure 1). In the absence of *S. oralis*, the allocation of *F. nucleatum* remained once again unchanged compared to the control (Figure 3A), but large and dense clusters of *S. mutans* populated the mass of the biofilm (Figure 3B). This visual observation is in line with the quantitative data, which indicate that *S. mutans* was detected in higher numbers when *S. oralis* was absent (Figure 1).

Discussion

Deciphering the interactions between species within biofilms is crucial for understanding the factors that ensure their stability, or those that drive changes that lead to dysbiosis. The Zürich *in vitro* biofilm models are useful tools to define such interactions of group of species [22–27], individual species [15, 16, 28–30], or their virulence factors [17, 18].

The present study employed the 6-species supragingival biofilm variant of this model to characterize the role of each one of its individual bacterial species (the

fungus *C. albicans* was not investigated). Among all tested species, omission of *F. nucleatum* from the biofilm composition resulted in a decrease of the total bacterial numbers. This may not be surprising as *F. nucleatum* is a "bridging" microorganism between early and late colonizing species [31, 32], and has a scattered distribution throughout the biofilm as shown in the present and earlier studies [15, 16, 29]. Hence, absence of *F. nucleatum* may compromise the structural integrity of the biofilm and hinder overall microbial growth, as evidenced by the reduced total bacterial numbers in the present study.

The most striking finding through was pertinent to the commensal *S. oralis*. Its absence from the biofilm resulted in the decrease in *A. oris* and increase of *S. mutans*, which densely populated the mass of the biofilm. An interrelationship between the commensals *A. oris* and *S. oralis* has previously been demonstrated in *in vitro* biofilm models [33–36]. Earlier studies have shown that co-cultivation of *S. mutans* with *S. oralis* significantly increases biofilm formation by *S. oralis*, compared to the respective mono-species biofilm [37], and that *S. mutans* is able to colonize much less efficiently on streptococcal biofilms than on *A. oris* ones [38]. These earlier observations are in agreement with this study showing that absence of *S. oralis* leads to overgrowth of *S. mutans* and suppression of *A. oris* in the biofilm. Collectively these findings denote that *S. oralis* suppresses *S. mutans* overgrowth, thus enabling the co-growth of commensal *A. oris* in the biofilm. Interestingly, absence of both *S. oralis* and *S. mutans* enabled *A. oris* to grow further and populate more densely the biofilm, denoting that *S. oralis* has a regulatory role on the growth of fellow early colonizer *A. oris*.

The molecular events behind these observations cannot be fully clarified under the present analytical approach. They may be due to lack of nutritional completion

between the two streptococcal species, or indeed lack of an antagonistic relationship between them, in which case it could account for active production of bacteriocins by *S. oralis* that inhibit *S. mutans*. It is less likely that hydrogen peroxide by *S. oralis* is involved in these events, as the biofilm was grown under anaerobic conditions when its production is expected to be limited. The reduced growth of *A. oris* in the absence of *S. oralis* may denote that the latter provides growth nutrients to the former, or that it protects it from a disadvantageous competition with *S. mutans*, potentially via the production of bacteriocins.

Streptococcus is the most predominant genus of the oral cavity, classified into four species groups [39]. Recent molecular studies have successfully identified new members of the oral streptococci [40, 41]. Despite their common genus classification, streptococci can exhibit diverse phenotypic [42] antigenic and genetic properties [43]. Oral streptococci have evolved to specifically colonize their human host, which they live predominantly in harmony with. They are the first microorganisms to colonize oral surfaces and are able to interact with many other oral species, hence they are instrumental in initiating formation of multi-species biofilms. However, given appropriate environmental conditions, some streptococci may enhance their virulence properties in the biofilm, and establish a dysbiotic state that leads to disease [44]. One classical such example in the oral cavity is the acidogenic and aciduric *S. mutans*, which is considered a highly contributing species to dental caries. Indeed, biofilms from caries–active dental sites harbor significantly greater proportion of *S. mutans* and lower proportion of *A. oris* than biofilms from the caries-free sites [45], whereas the presence of *S. oralis* is associated with a caries-free state [46].

In conclusion, the present study demonstrates that *S. oralis* may regulate the growth of *A. oris* and suppress the overgrowth of cariogen *S. mutans* in a biofilm

environment. This data supports the role of *S. oralis* as a commensal homeostasis-keeper in the biofilm, primarily by antagonizing *S. mutans*, thus preventing a caries-prone dysbiotic state. "Ecological" or "environmental" approaches for future preventive or treatment strategies for dental caries [47] may aim at ensuring the stability of *S. oralis* in the oral microbiome.

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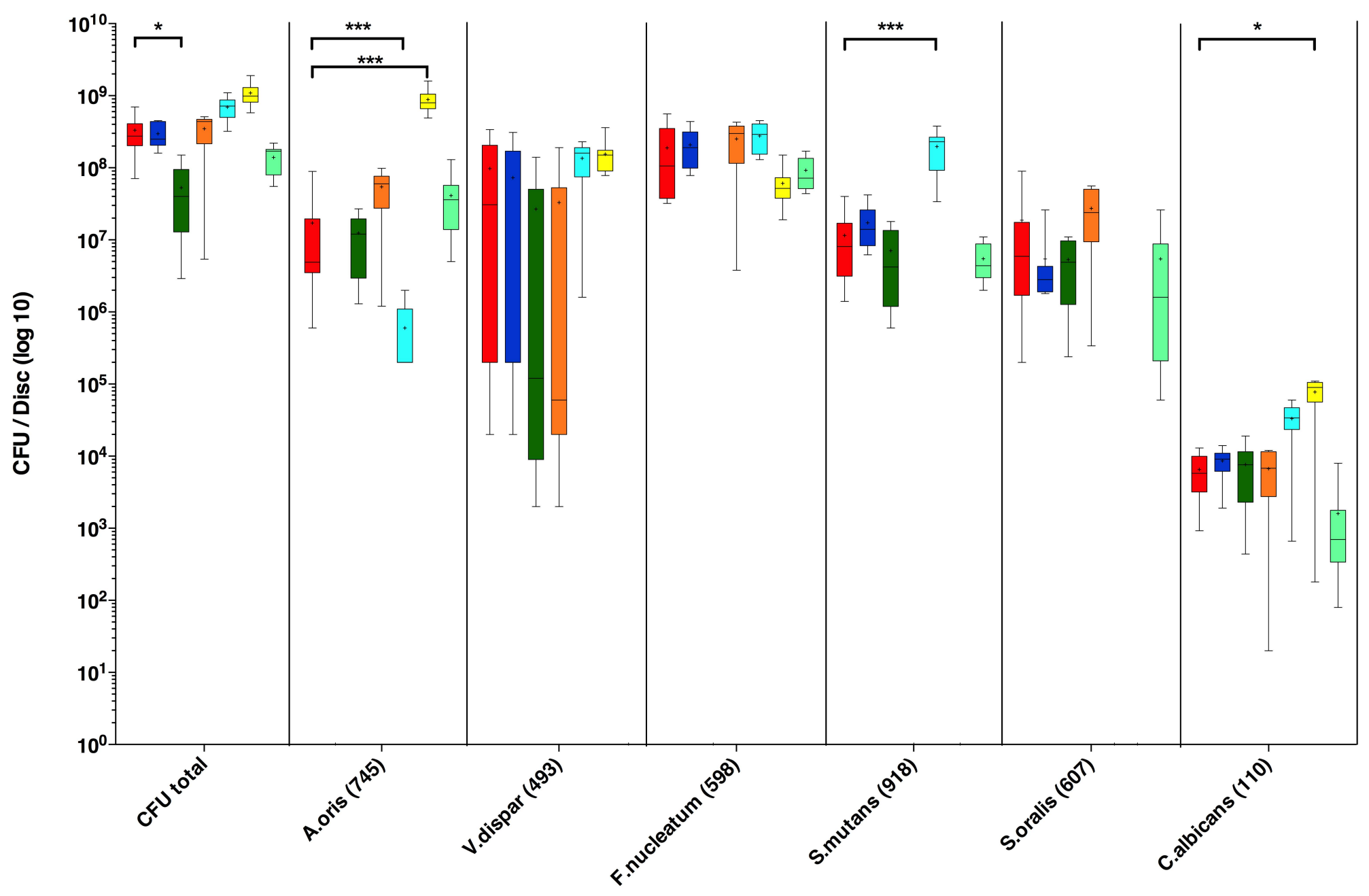
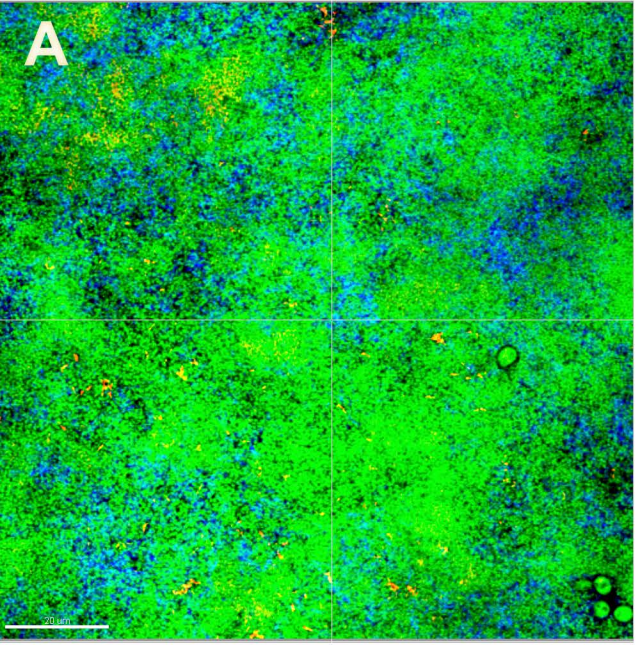
Figure Legends

Figure 1. Colony forming units (CFUs) of the 6 species control biofilm (control, red), or 5 species biofilm without *A. oris* (blue), or *F. nucleatum* (green), or *S. mutans* (orange), or *S. oralis* (light blue), or *V. dispar* (light green), or 4 species biofilm without *S. mutans* and *S. oralis* (yellow). Data derives from 3 independent experiments, in which every group was represented in triplicate biofilm cultures. Box plots represent the CFUs determined by selective agar plating, while horizontal lines indicate their median values. Undetectable values were ascribed the lowest detection limit value of the assay to allow for log transformation. Asterisks (*) represent significant difference compared with the control group ($P < 0.05$). Statistically significant differences compared with the control group are indicated with asterisks (* $P < 0.05$; *** $P < 0.001$).

Figure 2. Confocal laser scanning microscopy (CLSM) images of the 6 species (control) biofilm (A) and biofilm without *S. oralis* and *S. mutans* (B). Bacteria appear green due to DNA-staining using YoPro 1 iodide and Sytox green. Due to FISH staining with 16S rRNA probes Act476-Cy3 and FUS664-Cy5 *A. oris* and *F. nucleatum* appear red and blue, respectively. The biofilm base in the cross sections is directed towards the top view. Scales: 20 μm.

Figure 3. Confocal laser scanning microscopy (CLSM) images of the 6 species (control) biofilm (A) and biofilm without *S. oralis* (B). Bacteria appear green due to DNA-staining using YoPro 1 iodide and Sytox green. Due to FISH staining with 16S rRNA probes FUS664-Cy3 and STR405-Cy5 *F. nucleatum* appear red and streptococci blue, respectively. The biofilm base in the cross sections is directed towards the top view. Scales: 20 μm.


CFU / Disc (log 10)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
*
***
***
***
*
CFU total
A.oris (745)
V.dispar (493)
F.nucleatum (598)
S.mutans (918)
S.oralis (607)
C.albicans (110)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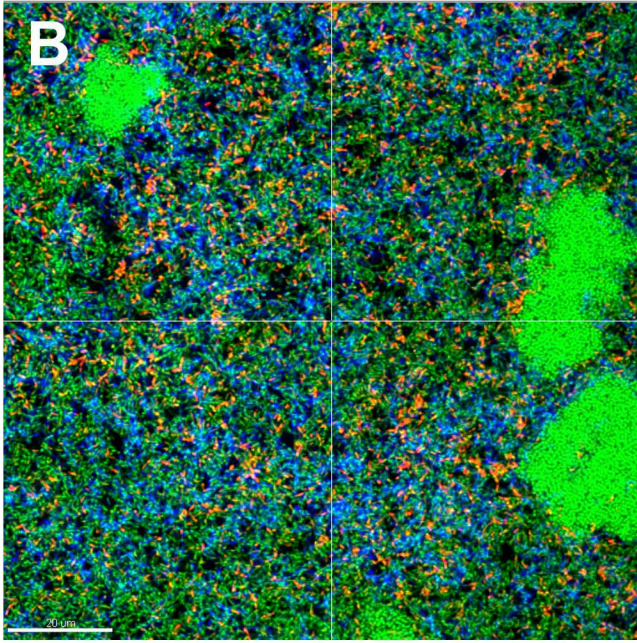
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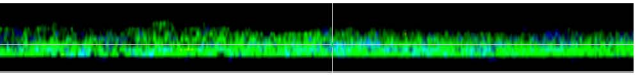

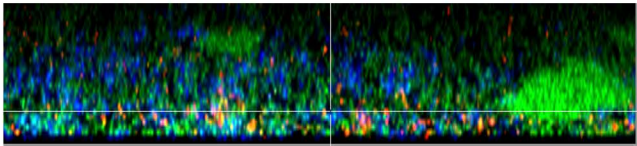

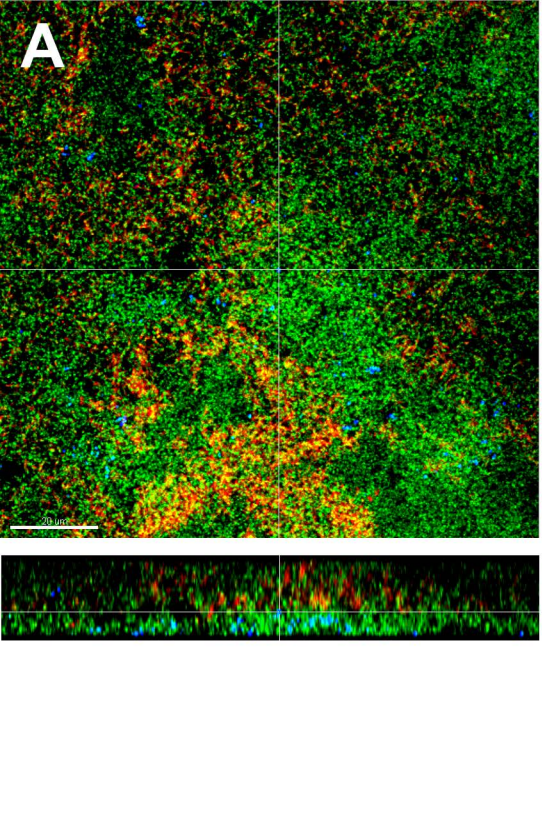
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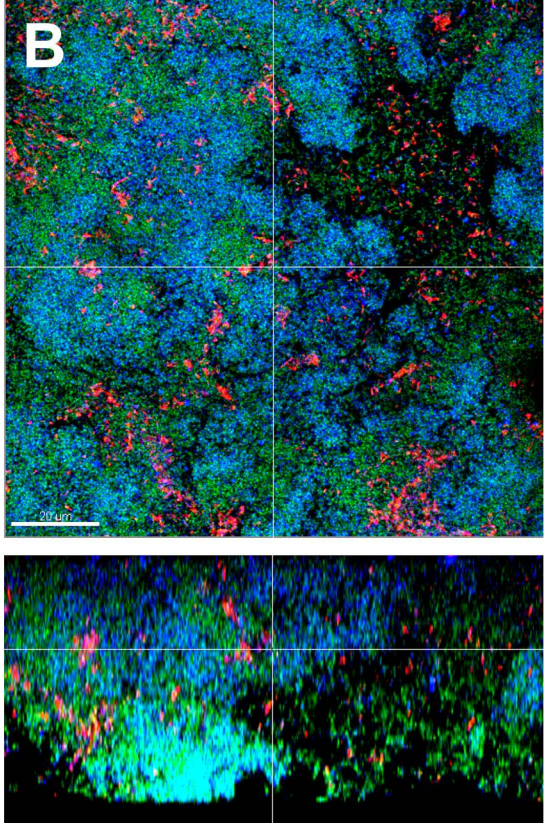
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